EXPERIMENTAL STUDIES ON THE VIRUS OF INFECTIOUS AVIAN ENCEPHALOMYELITIS

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Infectious avian encephalomyelitis, originally called "epidemic tremor of young chickens," is a disease only recently discovered, having been first described by Jones in 1932 (1). During the period extending from its discovery to the present time, the only additional account of it is given in a preliminary report by Van Roekel, Bullis, and Clarke in 1938 (2).

The writer's interest in this malady was aroused by the possibility of a relationship which might exist between the avian disease and equine encephalomyelitis, for recent findings indicated that the naturally occurring and the induced infection with the latter virus arise in game and domesticated birds. Furthermore, avian encephalomyelitis prevails chiefly in New England where, in 1938, an explosive epidemic of the equine disease in both horses and man broke out.[1] In view of this, and the fact that the incitant of the avian infection has hitherto had but scant study, it was thought desirable first to investigate its connection to the virus of the equine disease, and secondly, to learn something of the nature and properties of the avian agent, particularly of its place among the group of characteristic viruses.

Before reporting the results of our investigations, the main features of the disease as it occurs in nature should be summarized. The writer is indebted for information mainly to the comprehensive descriptions of Jones (1) and Van Roekel and associates (2).

Epidemiology.—Epidemics of the malady have attacked Rhode Island and New Hampshire Red, and Barred and White Plymouth Rock chickens, during the years 1930 to 1933 in New England, and recently it has been stated (2) that the disease is

[1] For literature and description of avian and equine encephalomyelitis infections and the epidemics of this disease in man, see Olitsky, P. K., in Brennemann, J., Practice of pediatrics, Hagerstown, Maryland, W. F. Prior Co., 1939, **4**, section II, chapter 7, pp. 35–42. To the references quoted there, another should be added (see reference 3). The latter article appeared when the present paper was being prepared for publication and referred to the clinical dissimilarity of avian and equine encephalomyelitis in birds.

becoming more common and is still most prevalent in the same region. There is a seasonal occurrence of epidemics in winter and spring months. The age of susceptibility to infection is from the time of hatching to 6 weeks, with the usual time of onset at 1 to 3 weeks of age. No epidemics are known to have occurred among adults (1). From 5 to 50 per cent of the number of a flock are sometimes affected. Jones (1) holds the opinion that there is no available evidence derived from experiments of mating diseased birds, that transmission of the incitant takes place through the egg, although Van Roekel (2) states that inoculation of eggs during their early incubation period yields a hatch of infected birds. The appearance of the disease in chicks one day old would favor the latter finding of egg-borne transmission of the disease. During three epidemics, contact experiments of normal birds caged with those having the malady failed to induce infection in the former (1).

Clinical Course.—In nature the first observable symptom is a dull expression of the eyes, and ataxia; the legs then become weaker, and the chicken inclines to sit on its haunches or walks on its hocks and shanks, frequently falling over. Tremor may occur, but not in all birds, and is usually limited to the head and neck. The weakness of the legs may progress to complete incapacity to move about, general debility increases and the chicken becomes prostrate and dies after a few days of illness. Or, tremulous birds without much ataxia may continue in this state with a general degree of health compatible with normal functioning (1), and in time the tremor and ataxia may disappear (2). The death rate in nature is unknown but Van Roekel estimates that losses from death and those killed may exceed 50 per cent of the total number in flocks.

Pathology.—As described by Jones (1) the lesions are only microscopic. In the brain and cord are accumulations of neuroglia cells clustered around capillaries, and perivascular infiltration about larger vessels. (In inoculated birds the perivascular infiltration is largely lymphocytic.) The condition of neurones is not mentioned but it is stated that degeneration of Purkinje's cells is often severe. Peripheral nerves are not involved. In the viscera, foci of infiltration with cells of the lymphoid series are found and are of two types: circumscribed and encapsulated, and irregular with ill defined borders. These lesions, which do not occur in inoculated birds (1), are said by Jones to be found in other avian pathological conditions, but those in the pancreas of chickens affected by encephalomyelitis are regarded as unusual. No inclusion bodies were seen in any of the cells (1).

EXPERIMENTAL

A strain of the infective avian agent in the form of a 10 per cent saline solution suspension of affected chick brain was sent to us through the kindness of Dr. Van Roekel.[2] It is with this material as the source that the following studies were carried out.

The experimental work hitherto performed with this infective agent, chiefly by Jones (1), indicated that no cultivable microorganism of etiological significance could be iso-

[2] We wish to acknowledge with pleasure our gratitude to Dr. Henry Van Roekel of the Department of Veterinary Science, Massachusetts Agricultural Experiment Station at Amherst, for his many courtesies and cooperation. The strain studied by Dr. E. E. Jones, to whom we also offer our thanks, was not available for comparative tests.

lated from active material. Bacteria-free suspensions of affected brain and cord could induce the experimental disease when given intracerebrally but not when introduced subcutaneously. Intraperitoneal injections of intestine, liver, spleen, or brain material, obtained from cases of encephalomyelitis, failed generally to bring about the clinical disease, so also did subcutaneous inoculation of suspensions of liver, spleen, pancreas, and gall bladder of affected birds. Failure to induce symptoms also followed the oral administration of intestinal walls and contents and of brain and cord. The intracerebral method and the use of brain or cord, or both, were considered the procedures of choice for uniform infection, the signs of which became clear after incubation periods of from 6 to 44 days, or as an average, 21 to 28 days.

It is worthy of note that in occasional instances inoculated birds would show none of the characteristic signs but at autopsy would reveal slight perivascular infiltration, "unequally stained" nerve cells, and rare small focal accumulations of glial cells. This occurred in one series in 2 of 45 birds receiving intracerebrally suspensions of brain or cord or both, and in an unstated but small number of chicks given suspensions of intestine, liver, spleen, and brain intraperitoneally or *per os*. Jones (1) ascribes this finding to the fact that these birds may have acquired the disease but in so light a form as to exhibit none of the typical signs. The observation takes on greater significance in view of the results to be reported later in this paper. Jones (1) also reported that the infective agent can pass through Berkefeld N and Seitz filters, and that it can survive when kept in 50 per cent glycerol for 69 days.

Van Roekel (2) employed throughout his studies, as did Jones (1), only one dilution (10^{-1}) of saline suspensions for transmission experiments. Van Roekel has confirmed the prior observations (1) of the infectivity of brain suspensions by means of intracerebral inoculations, and has carried out at least 42 serial passages. The infective agent was also recovered from organs other than the brain (2). Van Roekel states that inoculated birds could transmit the disease to normal chickens through cohabitation.

Methods and Materials

Chickens.—Rhode Island or New Hampshire Red chickens from 2 days to 6 weeks of age, mostly from 9 to 12 days old, were used throughout the present studies.

Those who have used young chickens for experimental work are aware of the difficulties encountered, first in the spontaneous development of a variety of ailments, and second, in maintenance of caged birds in a healthy condition. Practically all barriers were eventually surmounted so that experimental results could be properly interpreted. Our source of supply provided a good stock of 1 day old chicks which were kept in quarantine for 7 to 16 days before use. While in early stocks about 10 per cent of birds exhibited morbidity or mortality, later ones comprised chicks which were, as a rule, in a healthy condition over long periods of observation, due to the improvement in the method of caring for them. They were kept in cages supplied with a brooder or maintained constantly at a temperature of 33–34°C. They were given purina chick feed, placed in special containers, along with lettuce leaves and ample supplies of clean water. Care was taken to remove from the stock the occasional bird that showed weakness or disability. Some of these were passaged and others were killed and their tissues sectioned for pathological study. None showed evidence of infection with avian or equine encephalitis or other viruses. In this way a satisfactory stock was maintained and serious losses of chickens from accidental causes were avoided. The birds were anesthe-

tized with ether before inoculation or other procedures were carried out. Those up to the age of 7 to 10 days could easily tolerate a dose of 0.05 cc. intracerebrally; older birds received 0.1 cc. Other dosages will be indicated in the text.

At this point mention should be made of the fact that a small number, about 1 in 100, of chickens kept caged for prolonged periods, showed, usually after about 35 days of such confinement, evidence of weakness of legs ("cage paralysis") which may be confusing in establishing a diagnosis of encephalomyelitis unless confirmatory transfer to fresh chickens and a histopathological study of the nervous tissues are made.

Viruses.—The source of the avian virus has already been described but after the first few trials at passage of the Van Roekel strain, ordinary broth was substituted for saline as diluent. The Eastern equine encephalomyelitis virus was passaged intracerebrally almost continuously for about 6 years in white mice and fresh brain from affected animals was employed in the tests. The Western equine strain was passaged similarly in mice at less frequent intervals and during these intervals was kept in 50 per cent buffered glycerol in the cold. The virus suspensions were spun at 500 R.P.M. for 3 minutes, unless otherwise stated, and dilutions were made from the supernatant fluid.

The Experimental Disease.—The onset of the experimental disease resulting from inoculation of the avian virus by the various routes to be described later was noted within from 5 to 40 days, mostly in the period between 9 and 21 days. The first symptom to be observed was weakness of the legs; this sign became more pronounced with time, so that the chicken would squat on its shanks or fall on its side. No definite paralysis, either spastic or flaccid, was seen—the condition conformed more to a paretic state. The wings were not affected as a rule and convulsive seizures were absent. The eyes were dull; the birds kept up a weak chirp; and there was some loss of weight. In about 1 of 10 instances a fine or coarse tremor was observable, usually of the entire body, often of the head and neck alone, and occasionally only of the legs. In about 75 per cent of the number of affected birds the signs progressed, and on about the 3rd to the 5th day the chicken became prostrate and died; in about 90 per cent, death ensued within about a week after onset of symptoms. The remaining birds maintained for weeks a staggering or ataxic gait, some walking on their hocks and shanks, some continuously tremulous. During this prolonged period the general health was good. No definite change in the number or relation of the leucocytes in the circulating blood during the course of the affection could be discerned. The pathological changes in affected birds will be described in another section.

Controls and Contact Infection.—The experimental disease just described has not as yet been reproduced by the intracerebral inoculation into chickens of such materials as saline solution or suspensions of brain derived either from normal chickens or from those which were affected by ailments other than avian encephalomyelitis. No case of spontaneous occurrence of this malady has occurred among 592 normal chickens thus far observed. Similarly, we have recorded no instance of contact infection, either from association of normal with affected birds in the same cage (in at least 45 such normal chickens) or from ill birds in one cage to normal ones in another. In respect to absence of contact infection the results agree with those of Jones (1).

Relation of Avian to Equine Encephalomyelitis

For the study of the relation of the causal agent of avian encephalomyelitis to the equine virus, investigations were made in two directions:

host susceptibilities and immunological reactions. Since the avian disease prevails in New England where only the Eastern strain of the equine virus is found, most of the comparative tests were performed with that strain.

Host Susceptibility to Avian Infective Agent.—

Mice.—In the first test, seven 2 week old chickens were inoculated intracerebrally with 0.1 cc. of 10^{-1} dilution of brain, representing the fourth cerebral passage of the avian infective agent, or at least 10,000 minimal chicken cerebral infective doses. All developed the experimental disease, which was confirmed by passage and specific pathological changes. The same suspension was given to six 15 day old mice, 0.03 cc. into the brain of each and 1 cc. into the peritoneal cavity. No untoward effect was noted during the following 45 days. In the second test, similar to the first, 5 birds (controls) and 9 mice, 30 days old, received brain suspension representing the third cerebral passage of the avian virus. All the chicks developed encephalomyelitis but none of the mice revealed any sign of disease.

Guinea Pigs.—In the first test, 0.05 cc. of 10^{-5} dilution of avian virus in saline solution was specifically active in birds (saline solution was used since broth injected intracerebrally in guinea pigs is frequently toxic). 4 guinea pigs (average weight, 350 gm.) received 3 times this quantity (0.15 cc.) of 10^{-1} dilution of the same saline solution suspension but exhibited no signs of infection. In the second test, 7 control chicks were given 10^{-1} dilution of avian virus (at least 10,000 avian cerebral doses) and all developed encephalomyelitis, while 4 guinea pigs injected with 0.15 cc. of it intracerebrally plus 1 cc. intraperitoneally, failed to show any manifestations of disease, not even febrile reactions.

Rabbits.—Similarly, two series of tests performed with rabbits (1000 gm.) showed that of 2 which received 0.25 cc. of virus suspension intracerebrally (at least 10,000 avian cerebral doses) and 2 given this dose plus 5 cc. intraperitoneally, failed to respond with signs of infection.

Monkeys.—2 monkeys inoculated by the intracerebral route with 1 cc. of the same suspension as employed in the rabbits were also found resistant.

There exists, therefore, a limited range of host susceptibility to the avian active agent; only birds react to it. The ordinary laboratory animals, such as mice, guinea pigs, rabbits, and monkeys, are resistant to the effects of intracerebral inoculation of the infective agent—in certain instances to this method combined with intraperitoneal injection, even though large numbers of infective doses are used. Thus the avian virus differs from the equine; the latter is characterized by the susceptibility to the virus, cerebrally administered, of a remarkably wide range of animal species other than the horse (4) and of these, the white mouse and the guinea pig are particularly responsive.

Susceptibility of Chickens to the Equine Virus.—The virus of equine encephalomyelitis, on the other hand, is pathogenic for many varieties of birds, among which is the Rhode Island Red chicken (3,5), a species most commonly affected in nature by avian encephalomyelitis.

The results obtained with Rhode Island or New Hampshire Red chicks inoculated intracerebrally with mouse-passage Eastern equine encephalomyelitis virus confirms the findings of Tyzzer, Sellards, and Bennett (5), and of Van Roekel and Clarke (3) on the susceptibility of these birds to the Eastern equine virus. With the mouse-passage virus, however, large numbers of mouse cerebral lethal doses (M.C.L.D.) had to be employed to induce the experimental disease; it can be assumed that the chicken-passaged virus might be more infective. For example, 100 and 10,000 M.C.L.D. given intracerebrally failed to be disease-producing in all of 6 birds; 100,000 M.C.L.D. brought about death from encephalitis in 1 of 4 on the 4th day, and 1 million and 10 million doses, in all of 11, on the 2nd and 3rd day after intracerebral inoculation (two experiments). When the mouse-passaged Western strain of the equine virus was used, cerebral injection of 1 million M.C.L.D. induced signs of encephalitis and death on the 3rd day in only 1 of 4 chicks; fewer number of doses showed no such effect. In this connection, reference should be made to earlier work which indicated the relative resistance of chicks to the Western virus which was also passaged in rodents for a long period of time (6). Moreover, Shahan, Giltner, and Schoening (7) have reported that the two strains of equine virus are each infective for particular species of animals.

The signs in birds affected by the equine virus were distinctive and unlike those of experimental avian virus disease. The onset was sudden, with prostration, continuous staccato, harsh cries; then coma and terminal convulsive movements of the wings and legs. Death followed rapidly, usually on the first day of definite signs. At autopsy, no constant gross lesions were visible, either in the viscera or in the central nervous system. Microscopically, the latter exhibited throughout the brain and cord extensive perivascular infiltration with lymphocytes and large mononuclear cells, some plasma cells and some polymorphonuclear cells. Associated with this infiltration was proliferation of the adventitial cells. The endothelium was swollen, and here and there a vessel exhibited disintegration of its media. Active neuronal necrosis associated with neuronophagia was evident, as well as proliferation of glial elements throughout the brain. Isolated glial accumulation and disorganization of the ependymal cells occurred. The meninges were not much involved except for the vessels therein which exhibited the marked perivascular infiltration and adventitial proliferation. No definite intranuclear inclusion bodies, characteristic of equine virus infection, could, however, be discerned.

The action of the virus of equine encephalomyelitis in the birds is distinctive and differs from that of the avian virus not only in the course of the experimental disease but also in its signs and in the induced pathological changes. Moreover, Rhode Island and New Hampshire Red chicks are susceptible to the Eastern equine virus, but with the stock strain available, continuously passaged in mice for several years, only large numbers of infective doses (1 million or more M.C.L.D.) are disease-producing. Finally, such birds are practically resistant to the Western equine strain, mouse-passaged for a long period of time.[3]

[3] For an adaptation of equine encephalomyelitis virus in a somewhat reverse direction, namely, pigeon-passaged virus decreasing its relative pathogenicity for guinea pigs, see Traub, E., and TenBroeck, C. (*Science*, 1935, **81**, 572).

Immunity Tests; Active Immunity.—The first step was to determine whether chickens having had the clinically apparent, experimental avian disease develop resistance to reinoculation with the same virus.

In view of the fact that only a few birds survive an attack of the experimental malady, the following test was restricted to a small number of convalescents. 4 chickens, inoculated cerebrally with the avian virus and selected because they showed a non-progressive ataxia, or ataxia associated with tremors, enduring from 11 to 31 days, before the immunity test, received in the brain 10^{-1} dilution of the avian virus representing at least 10,000 minimal infective chick cerebral doses. They continued since then with their clinically stationary course, or even with some slow improvement of health, whereas control normal birds of the same age receiving cerebrally decimal dilutions (10^{-1} to 10^{-5}) of the same virus suspension, came down with the characteristic encephalomyelitis.

It would appear, therefore, that affected birds in a non-progressive stage of experimental avian encephalomyelitis develop a resistance to cerebral reinoculation of the avian virus. At the present time we have not gone beyond this point, into the field of cross active immunity tests with the equine virus, because (*a*) the Eastern strain of the latter, when active, invariably induces in chickens lethal infections, thus leaving no survivors for a test, and the mouse-passage Western strain produces in them no clinically apparent disease regularly, even with large numbers of infective doses; (*b*) laboratory animals (especially mice) are resistant to infection with the avian virus; and finally, (*c*) survivors after inoculation of chickens with the avian virus, in a proper clinical state for reinoculation with the equine infective agent, were difficult to collect. Further work in this problem is still being carried on. Consideration should be given to the possibility of studying active immunity to the avian infective agent and cross reactions with equine virus by means of immunization with formolized vaccines.

Serological Immunity.—Recourse was had to serum-protection tests which were performed on the basis of the neutralizing capacity of antisera when the serum and virus were mixed and the mixture injected immediately into the brain of chickens. It was necessary, of course, first to determine whether antiviral substance develops in the serum of birds which have been affected by the experimental avian disease.

Serum was collected and pooled from 4 birds which had the experimental avian disease and failed to react to an intracerebral test dose of at least 10,000 minimal infective cerebral doses of the virus. They were bled 42 days after this test dose was given. The serum showed protection against 10 to 100 such doses, if 10^{-6} dilution of virus is taken in this experiment as the limit of its infectivity.

Thus serum derived from birds having the experimental avian disease is found to contain antiviral bodies. It may be possible that the titer of

neutralization could be increased by the introduction of modifications in the test, such as incubation of the mixtures before injection, but the neutralizing effect, as shown in Table I, is definite. The production of more potent antiserum by immunization of rabbits or other animals, as is done with certain other viruses, is also to be considered in future work on this problem.

The next step was to test the capacity of this antiserum to avian virus to protect against the effects of the Eastern equine virus. As indicated in the preceding paragraphs, chickens are susceptible only to high multiples

TABLE I

Neutralizing Antibodies in Chickens Convalescent from Experimental Avian Encephalomyelitis

(0.05 cc. of mixture of equal parts of dilution of avian virus and undiluted serum or broth injected intracerebrally in 3 day old chickens)

Virus mixed with	Final dilution of virus	Number of chickens injected	Number dead of encephalomyelitis	After incubation period
				days
Broth (control)	10^{-3}	5	5	12–21
	10^{-4}	5	3*	19–26
	10^{-5}	5	3	13–19
	10^{-6}	5	2	12–24
Convalescent serum	10^{-2}	5	5	14–19
	10^{-3}	5	5	16–29
	10^{-4}	5	2	24–29
	10^{-5}	5	0	—

* 1 of 3 survived with symptoms of paresis and ataxia.

of lethal doses of the available mouse passage virus; hence, the test was made on the mouse which is so highly susceptible. Furthermore, the intraperitoneal route in 15 day old mice was used for inoculation of serum-virus mixtures, a procedure shown to be more highly sensitive for detection of neutralization than intracerebral inoculation in mice of all ages (8).

Control mice were injected intraperitoneally with suspensions of the Eastern equine virus diluted decimally in broth from 10^{-3} to 10^{-8}. The animals succumbed to all dilutions. With similar inoculations of mixtures of equal parts of undiluted antiserum to avian virus—the sample just described—and decimal dilutions of Eastern equine virus, again mice receiving all dilutions developed encephalomyelitis. An additional similar neutralization test was performed in mice with the anti-avian virus serum and the Western equine virus. Here also no evidence of protection was obtained.

Conversely, antiserum to Eastern equine virus derived from immunized rabbits, and

of a capacity to protect against 100,000 to 1,000,000 intraperitoneal mouse lethal doses by the mouse intraperitoneal method (8) and against 10 intracerebral doses by the mouse intracerebral test, was examined for its neutralizing effect in birds on the avian virus. In a test similar to the preceding one no evidence of neutralization was found.

One may conclude from the foregoing series of experiments that (*a*) an attack of experimental avian encephalomyelitis leads to development of resistance to the effects of reinjection of the virus into the brain; (*b*) the serum of such birds acquires neutralizing antibodies, as shown by the method here employed, namely, intracerebral injection of serum-virus mixture; (*c*) the antiserum to avian virus, collected from those chickens in the quiescent stage of the experimental malady, does not protect against the Eastern equine virus in tests performed by the intracerebral route; nor does antiserum to the equine virus protect by this route against the avian infective agent. The experiments in immunity and on pathogenesis of the viruses in different hosts offer sufficient evidence that the two infective agents, the equine and the avian, are characteristically separate and distinct.

Properties of the Avian Infective Agent

As the studies on the properties of the incitant of the avian disease progressed, it became apparent that they were common to those of several other filtrable viruses of smaller size.

Transmissibility.—Thus far, at least 12 brain to brain, consecutive serial passages of the avian virus have been successfully carried out. Infection can be produced as a rule in all chicks inoculated intracerebrally with decimal dilutions of the virus from 10^{-1} to 10^{-3}; 10^{-4} and 10^{-5} dilutions are effective in the majority of birds so injected, and with 10^{-6} dilution, in 5 different experiments, 2 of 5, 1 of 3, 1 of 3, none of 3 and none of 4 chickens, exhibited the experimental disease after cerebral inoculation.

Centrifugation.—In the preparation of stock filtrates for gradocol membrane, ultrafiltration studies, it was found that spinning a virus suspension (1:10) at 5400 R.P.M. for one hour in an angle centrifuge yielded a sediment which when reconstituted to 1:10 in broth induced encephalomyelitis in all of 4 birds cerebrally inoculated with it. The supernatant fluid was also infective to that degree in which 10^{-5} dilution of it induced disease in 3 of 4 birds. For ultrafiltration studies, centrifugation in Bauer and Pickel's open air centrifuge (9) at 12,000 R.P.M. for one hour can also yield supernatant fluid satisfactory in virus content for membrane filtration.

Filtration.—Table II summarizes the results of filtration experiments. It will be noted that the virus is capable of passing readily through Berkefeld V and N candles and Seitz 1 and 2 disc filters.

Size.—In preliminary experiments performed with the cooperation of Dr. J. H. Bauer (the details of which will be published elsewhere) it was found that chicken brain virus suspended in broth passed through gradocol membranes of 73 mμ and larger, average

pore diameters. Further attempts are, however, being made to determine whether the virus can transverse still finer membranes.

Bacterial Cultures.—The filtrates just described were free from the presence or growth of bacteria of the ordinary species, as revealed by the negative findings in film preparations of active tissues of the central nervous system, or in stained sections of the latter, and by failure of fluid and solid media to show growth. In addition, cultures made after the methods of Sabin (10) for detection of pleuropneumonia-like microorganisms, were also negative.

Resistance to Glycerol and Drying.—The infective agent, as contained in affected chick brain, and kept in 50 per cent glycerol for at least 88 days (longer time not as yet studied)

TABLE II

Filtration of Avian Encephalomyelitis Virus

Experiment	Suspensions Horizontal centrifugation	Intracerebral dose	Number of chickens inoculated	Number showing encephalomyelitis	Incubation period
		cc.			*days*
I	Unfiltered 10^{-1} dilution 500 R.P.M. × 3 min.	0.05	4	4	8–13
	Same; filtered Berkefeld V	0.05	4	4	11–20
	Same; filtered Berkefeld N	0.05	4	4	10–16
	Same; filtered Seitz 2 discs	0.05	4	4	7–20
II	Unfiltered 10^{-1} dilution 500 R.P.M. × 3 min.	0.1	4	4	8–12
	Same; filtered Seitz 1 pad	0.05	6	6	6–14
III	Unfiltered 0.5×10^{-1} dilution 2500 R.P.M. × 10 min.	0.1	4	4	8–13
	Same; filtered Seitz 1 disc	0.1	4	2	20–25
IV	Unfiltered 0.5×10^{-1} dilution 2500 R.P.M. × 10 min.	0.05	6	6	10–13
	Same; filtered Seitz 1 disc	0.1	6	4	11–21

was still capable of inducing the characteristic disease in 4 of 5 birds, when the glycerolated brain, diluted 10^{-1}, was given cerebrally. Similar material frozen and dried in the lyophile apparatus of Flosdorf-Mudd was found active after at least 68 days, the longest time thus far tested.

With respect to the properties thus far investigated, the infective agent of avian encephalomyelitis conforms in general to the similar characteristics of the established viruses of smaller size.

A study was then undertaken of the pathogenicity of the avian virus when it is inoculated by different peripheral routes.

Pathogenicity by Different Routes of Inoculation

Table III summarizes the results of inoculation of the avian virus by various routes. There were four separate experiments and the virus em-

TABLE III

Effect of Inoculation of Virus by Various Routes

Experiment	Route	Amount given of 10^{-1} dilution	Minimal infective cerebral doses	Number of birds inoculated	Number showing encephalomyelitis	Incubation period	Remarks
		cc.				*days*	
1	IC	0.05	Not tested	4	4	10–15	Source of material = 3 brains pooled, derived
	IP	0.5	" "	4	1	12	from birds ill 1 day; in Experiment 2, 3 brains,
	SC	0.5	" "	4	1	14	birds ill 1–2 days; in Experiment 3, 3 brains,
	ID	0.2	" "	4	2	8–11	birds ill 1–4 days, and in Experiment 4, 3
	IN	0.1	" "	4	0	—	birds, ill 1–9 days
2	IC	0.05	10,000	4	4	12–16	Seitz 1 disc filtrate
	IV	0.1	"	2	1	17	" " "
	IO	0.03	"	4	0	—	Unfiltered suspension centrifuge 2500 R.P.M. × 10 min.
	ID	0.2–0.3	"	4	2	13–17	" "
	PO	0.5	"	4	0	—	" "
	IM	0.2	"	4	1	17	" "
3	IC	0.05	10,000	4	4	9–11	Suspension centrifuged 500 R.P.M. × 3 min. (unfiltered) as in Experiment 1
	IN	0.1	"	6	0	—	
	IS	0.02	"	5	1	20	
	IM	0.2	"	3	1	14	
	PO	0.5	"	6	0	—	
4	IC	0.05	1000–10,000	4	4	9–20	Suspension centrifuged 2500 R.P.M. × 10 min. (unfiltered)
	SC	0.5	"	6	3	13–28	
	ID	0.2	"	6	0	—	
	IV	0.2	"	3	1	15	

IC = intracerebral; IP = intraperitoneal; SC = subcutaneous; ID = intradermal; IN = intranasal (0.05 cc. for each nostril, instillation); IV = intravenous; IO = intraocular (vitreous); PO = *per os* (through stomach catheter); IM = intramuscular; IS = intrasciatic.

ployed in all was contained in cerebral tissue and was derived from birds in the early stages of the experimental disease, with one exception, as noted

in Experiment 4. It will be observed that the intracerebral injection was followed by uniform and invariable development of the disease. In the section on dilution of virus, already mentioned, will be found additional evidence to show that the regularity of response to cerebral injection depends on the number of infective doses in the inoculum.

After peripheral inoculation, however, no such uniformity of results was seen. In two experiments in which 10 birds received the virus (as much as 10,000 minimal chicken cerebral infective doses) by means of nasal instillation, none reacted with signs of infection. So also 10 birds given the virus in the proventriculus by means of a catheter and 4 receiving the virus intraocularly (vitreous) failed to develop the disease. Yet small numbers of chickens could be brought down with experimental encephalomyelitis by other peripheral routes of inoculation, as for example, by intraperitoneal, subcutaneous, intradermal, intravenous, intramuscular, and intrasciatic injections. The positive results in these series were confirmed by successful transfer of the induced disease to normal birds (brain to brain transfer) or by the presence of characteristic pathological changes, or by means of both methods. It should be noted that as a rule the affection brought about by means of peripheral inoculation followed after a longer incubation period than that after intracerebral. In general, more work is necessary to disclose whether the virus, after peripheral introduction, persists in all or selected tissues of birds showing no clinically apparent disease, whether the hosts develop then antiviral bodies, and finally, whether old animals reported as being resistant to natural or induced infection (1, 2) can yield virus from their tissues or have antibodies in their serum. No definite indication could be found of varying susceptibility of chickens of the different ages thus far employed in this investigation, that is, from 2 days to 6 weeks, to central or peripheral inoculation of virus.

Persistence of Virus in Ataxic Birds.—One experiment, in which the brain of a chicken which had non-progressive ataxia, associated with tremor, lasting for 24 days—the result of cerebral administration of avian virus in 1:20 dilution—revealed the presence of virus in 10^{-1} but not in a higher dilution of its cerebral tissue.

Search for Virus in the Blood

The question arose whether virus circulates or multiplies in the blood of birds which are (*a*) in the acute phase of the experimental disease, or (*b*) in the period of its incubation.

The following experiment was designed to test this point.

Three birds, ill 1, 3, and 4 days, respectively, with experimental avian encephalomyelitis, brought about by intracerebral inoculation of virus, were bled from the heart and clotting prevented by the addition of 1:500 heparin. The brains of the etherized chickens were removed and pooled. 5 birds were immediately inoculated by the cerebral route each with 0.05 cc. of the pooled, undiluted blood; none of them showed disease. On the other hand, the brains of the same chickens, pooled and given cerebrally to fresh birds, were pathogenic in dilutions of 10^{-1} to 10^{-5}.

TABLE IV

Virus in Blood before Clinically Apparent Experimental Disease Developed

Chicken No.	Incubation period	Death from encephalomyelitis (time after 1st sign)	Time before 1st sign when bled	Blood transferred to chicken No.	Result
	days	*days*	*days*		
7-28	9	2	1	7-38	Well
				7-39	"
				7-40	"
				7-41	"
7-29	13	4	4	7-42	"
				7-43	"
				7-44	"
7-30	13	4	4	7-45	"
				7-46	"
				7-47	"
7-27	15	5	5	7-48	"
				7-49	"
				7-50	"
7-31	9	8	Control not bled	Not transferred	—
7-32	17	3	" " "	" "	—
7-33	9	Survived; residual ataxia and tremor	" " "	" "	—

In the next test, 7 birds received 10^{-1} dilution intracerebrally of 3 brains derived from chickens in the 2nd day of the induced illness and pooled. It was planned to bleed 4 of the birds at varying periods before the first clinical sign was evident and to retain the remaining 3 as controls. From each of the first group heparinized blood (about 5 cc.) was obtained by cardiac puncture and 0.05 cc. of this undiluted blood was immediately injected into the brain of each of 3 or 4 fresh chickens for each sample. These latter birds recovered from the bleeding and were permitted then to run their natural course, as were the second group of 3 which were not bled and served as controls for the determination of viral effect. Table IV summarizes the experiment.

It would appear from the foregoing tests that during the early stages of the clinically outspoken experimental disease, virus was not detected in the circulation. Nor could virus be found in the blood during the incubation period extending from 1 to 5 days before the first clinical sign of the infection was manifest. If virus had been present during either phase of the disease, and possibly missed by the tests as performed, even then such small undetectable amounts would be indicative of its failure to multiply in the blood stream.

Pathology

No definite distinction could be made in the general pathological picture of the experimental disease whether it was induced by inoculation of virus into the brain or peripherally. The macroscopic examination of chickens in any of the stages of the malady revealed no changes; microscopically, however, lesions were observed in the central nervous system and in the viscera, with some variations, depending on whether the tissues examined were derived from the acute or the chronic, quiescent phase of the ailment.

Central Nervous System.—In the first few days of the clinical signs, one observes no definite meningeal reaction, the spotty areas of involvement in the coverings of the brain are related to the vessels therein which showed the perivascular infiltration, to be described. The lesion most commonly met with, and the most striking, is neuronal degeneration, involving the cells throughout the central nervous system, but more extensive in the pons-medulla and in the anterior horn cells of the spinal cord, especially in the lumbosacral enlargement. The neuron first becomes rounded in outline and enlarged or swollen; the nucleus also increases in size. The next stage consists of an eccentric placement of the nucleus, which may reach the very limits of the cell membrane, giving the impression in certain instances of the nucleus being pressed upon by the contents of the other cell constituents. At the same time, there is active tigrolysis and clearing of the cytoplasm of Nissl substance. There is at first only a halo of Nissl bodies about the periphery of the perikaryon; later, even this thin line of granulation disappears, along with the nucleus, so that finally the perikaryon is stained homogeneously pink to red (in sections colored by eosin- or phloxin-methylene blue or by Giemsa's method). Certain of the cells in this latter condition exhibit a bright red, round mass, which is not the nucleus or nucleolus, about the size of a red blood corpuscle, which stands out in sharp contrast to the reddish, even or smooth background. In advanced stages, only faint pink shadows of the attacked neurons are left and now and again the cell completely disappears. It is noteworthy that in early cases of the affection there may occasionally be no other sign of involvement of the nervous tissue, especially no inflammation or secondary reactions. This is more likely to happen in the anterior horns of the cord. The process resembles Nissl's degeneration, or the axon reaction, that is, the neuronal degeneration which follows section of the axon, or neuritis due to inflammation or toxic action, or after stovaine anesthesia (11), or even after artificial stimulation (Spielmeyer, 11) (Figs. 1 to 3). The intensity of the reaction is worthy of mention. The usual picture of neuronal necrosis associated with varying degrees of

neuronophagia, characteristic of several neurotropic viruses, is apparently absent here. In the early stages of the malady, most of the Purkinje cells are as a rule found to be well preserved (Fig. 4). Here and there some cells show degeneration and in exceptional cases, especially late in the affection, considerable destruction of them occurs (Fig. 5).

Another change in the nervous tissue is the perivascular reaction which may reach an extraordinary degree (Figs. 4 to 6). This reaction pervades the entire brain, especially in the cortex and pons-medulla and cerebellum (Figs. 4 and 5). The white and gray matter of the cord is relatively free, however, from such involvement. The perivascular infiltration is mostly by lymphocytes, with an occasional large monocytic cell, and the dense collar may comprise a depth of 10 or more rows of such cells (Fig. 6). Vascular thrombosis and damage to the walls are not ordinarily found. In the early disease when the perivascular lesions are present, neuronal degeneration is, as a rule, concomitant; the reverse does not hold generally; only in certain few instances does neuronal degeneration alone exist. In chronic (quiescent) ataxic and tremulous birds, some indications of neuronophagia by glial elements are visible, also small accumulations of such cells, and rarely satellitosis. These lesions are, however, not common. Such chickens as were examined at about the 3rd week of illness, would show only perivascular lesions, mostly in the brain, the process of neuronal regeneration being then fairly well completed.

The ependymal lining cells and the peripheral nerves appear to be spared from attack. In occasional instances, neuronal degeneration of the axon reaction type can be seen in the spinal ganglia. Demyelinization is not noted.

The changes just described are found associated with the clinically apparent form of the disease. In similar examinations of the central nervous system of more than 20 chickens of the same stocks used as controls, uninoculated and inoculated with materials other than the avian virus, no such lesions could be discerned. On the other hand, all of more than 40 birds that have exhibited to the present time characteristic clinical signs have revealed the pathological conditions as described.

Other Organs.—No gross changes are found in the other organs of the body, but certain histopathological changes can be seen.

As is known, the chicken utilizes for its lymphatic glandular system small islands of lymphoid tissue, sometimes located in the parenchyma and often associated with blood vessels. They are found in all organs and in the brain as well, where they are localized in the choroid plexus. Under the influence of avian virus infection these areas become markedly hyperplastic and show, apart from lymphocytes, few monocytes and myelocytes and some cellular debris (Figs. 7, 8, and 9). Practically all organs are so involved, but especially the liver, the pancreas, and the spleen. In the heart, the lymphocytic cells may arrange themselves longitudinally between muscle fibers, and probably this arrangement is the consequence of the vascular distribution of the lymphoid tissue. In other viscera the hyperplastic lymphoid areas may show a delicate circumferential membrane, or capsule.

In nervous or non-nervous tissue the polymorphonuclear leucocyte does not enter into the pathological reactions. Thus, a distinction is created between these lesions and those induced by several other viruses. No definite inclusion bodies could be detected and film preparations of affected tissue or centrifugalized sediments failed to reveal elementary bodies.

To summarize, the main pathological reactions found in experimental avian encephalomyelitis relate to (*a*) neuronal degeneration in the brain and cord resembling that known as axon reaction (or "primary or retrograde" degeneration); (*b*) perivascular lymphocytic infiltration, more pronounced in the brain and cerebellum than in the cord; (*c*) hyperplasia of the normal lymphoid islands in other organs; and (*d*) absence, as a rule, of polymorphonuclear cells in any of the pathological reactions.

DISCUSSION AND SUMMARY

The results of investigations thus far carried out on experimental avian encephalomyelitis indicate that the virus of this newly described disease conforms to the group of definitely established viruses. It was essential to determine its taxonomy since the only prior record of its study (1) defines the infective agent as a virus because the usual cultural attempts failed to reveal a visible microorganism to be identified with it, and because the transmissible agent passed through Seitz and Berkefeld N filters. At the present time such determinants fail completely to satisfy the criteria for defining a virus and their acceptance would lead to the inclusion of certain filtrable microbic agents, difficult to reveal except by special cultural procedures, as viruses (10).

The virus of avian encephalomyelitis is distinct from that of equine encephalomyelitis and is clearly a virus *sui generis*. The striking feature of its properties is its narrow range of host susceptibility—only the avian species are responsive to inoculation; ordinary laboratory animals are apparently resistant, even to large numbers of chicken cerebral infective doses. In addition, it is probable that its size is in the range of that of the equine virus. Studies also reveal that the virus is not easily sedimented by centrifugation (that is, at 5400 R.P.M. for one hour in the angle centrifuge and at 12,000 R.P.M. for one hour in the open air centrifuge) and is resistant to the action of glycerol and to drying. It is readily filtrable through Seitz one and two disc filters, through Berkefeld V and N candles, and is active in dilutions in broth up to 10^{-6}. It passes through gradocol membranes of 73 mμ average pore diameter at least (the end-point has not as yet been definitely determined).

An attack of the experimental disease leads to development of resistance to reinoculation and of antibodies in the serum. Old birds are reported as being refractory to infection, both in nature and in the laboratory (1, 2). Whether this resistance in mature animals is due to earlier exposure to infection, or to the development of structural or physiological barriers to invasion by the virus, remains still to be determined.

Under experimental conditions, the route by which the virus acts uniformly to induce disease is the intracerebral. Yet in certain instances other peripheral ways of inoculation such as the intraperitoneal, subcutaneous, intradermal, intravenous, intramuscular, intrasciatic, may also be effective. Thus far, in limited experiments, feeding, or instilling nasally, or injecting into the vitreous body the infective agent has been ineffective. Whether the viral progression is axonal from peripheral sites is still to be determined; as should be also the question whether it multiplies in any of the organs other than the central nervous tissues. The virus was not detected in the blood during the period of incubation or during the acute phases of the experimental disease. So that unless it is found that other animals harbor the virus, or that still other sources of it exist as yet not disclosed, it is not likely that the disease is disseminated by a blood-sucking insect. The actual portal of entry and the factor in the spread of the disease in nature is still obscure, since the evidence here presented is still too incomplete to elucidate these problems.

The pathological lesions induced are of interest. The neuronal reaction resembles that brought about by axonal disturbance (axon reaction, Nissl's or retrograde degeneration). The question may well be asked whether there may not be here an initial injury by the virus to the axonal process of the neuron, which in turn induces the retrograde changes in the cell body. This has as yet to be studied, as well as the possibility of viral progression along an axonal route with or without concurrent multiplication. The significance of the second major lesion in the central nervous system, namely, the generally marked perivascular reaction, is also still to be determined.

Finally, the only observable and histopathological change in organs other than the central nervous tissues (in which we have not as yet noted the change) is in the hyperplasia of the normally present lymphoid islands. One is impressed by the prodigious numbers of lymphoid elements surrounding the vessels of the central nervous system and the question here is whether these hyperplastic areas serve as depots to supply the cells for this perivascular reaction.

CONCLUSIONS

The transmissible causal agent of infectious avian encephalomyelitis of young chickens is a virus with traits of its own and is distinct from that of equine encephalomyelitis. The results of experiments provide a basis for the identification of the avian virus. The criteria relate to its antigenicity and serological reactions; to its size and various other physical properties;

to its pathogenicity by various routes of inoculation; and to its capacity to induce specific histopathological lesions.[4]

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EXPLANATION OF PLATES

All tissues were fixed in Zenker's acetic solution and sections stained with eosin-methylene blue.

PLATE 43

FIG. 1. The retrograde neuronal degeneration is shown in different stages. From avian virus infected chicken (3-25) intracerebrally inoculated, incubation period 14 days, clinical signs 3 days. Third cerebral passage Van Roekel strain. × 500.

FIG. 2. Showing extent of neuronal involvement. Chicken 4-80 intracerebrally inoculated with 1:100,000 dilution of virus; incubation period 17 days; sick 4 days. × 250.

FIG. 3. Neuronal degeneration in cord. No vascular lesions. Chicken 3-53 in fifth cerebral passage of Van Roekel strain. Incubation 11 days and paretic signs for 3 days. × 250.

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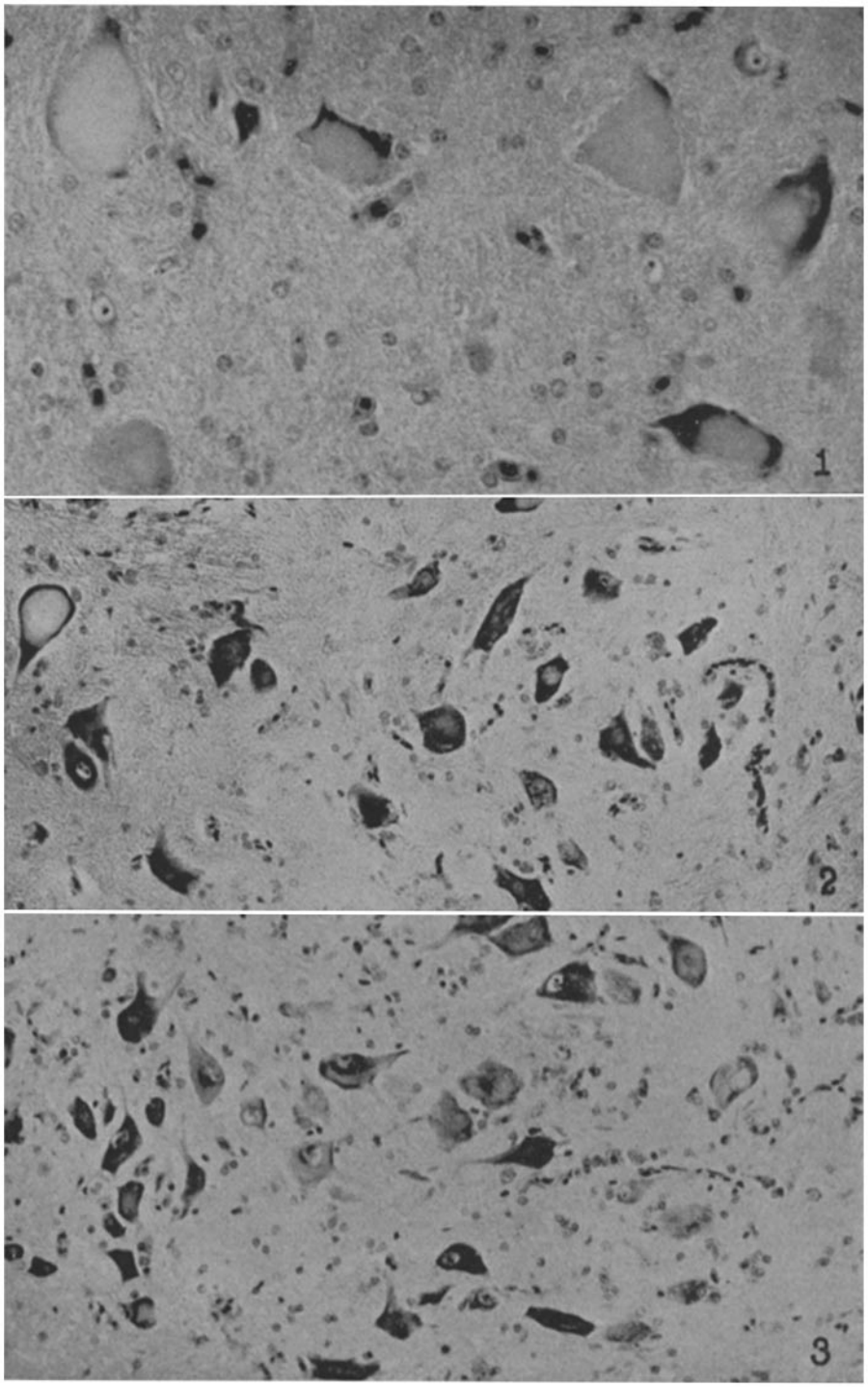
[4] The writer wishes to thank Mr. Peter Haselbauer for his valuable technical assistance.



Photographed by Joseph B. Haulenbeek

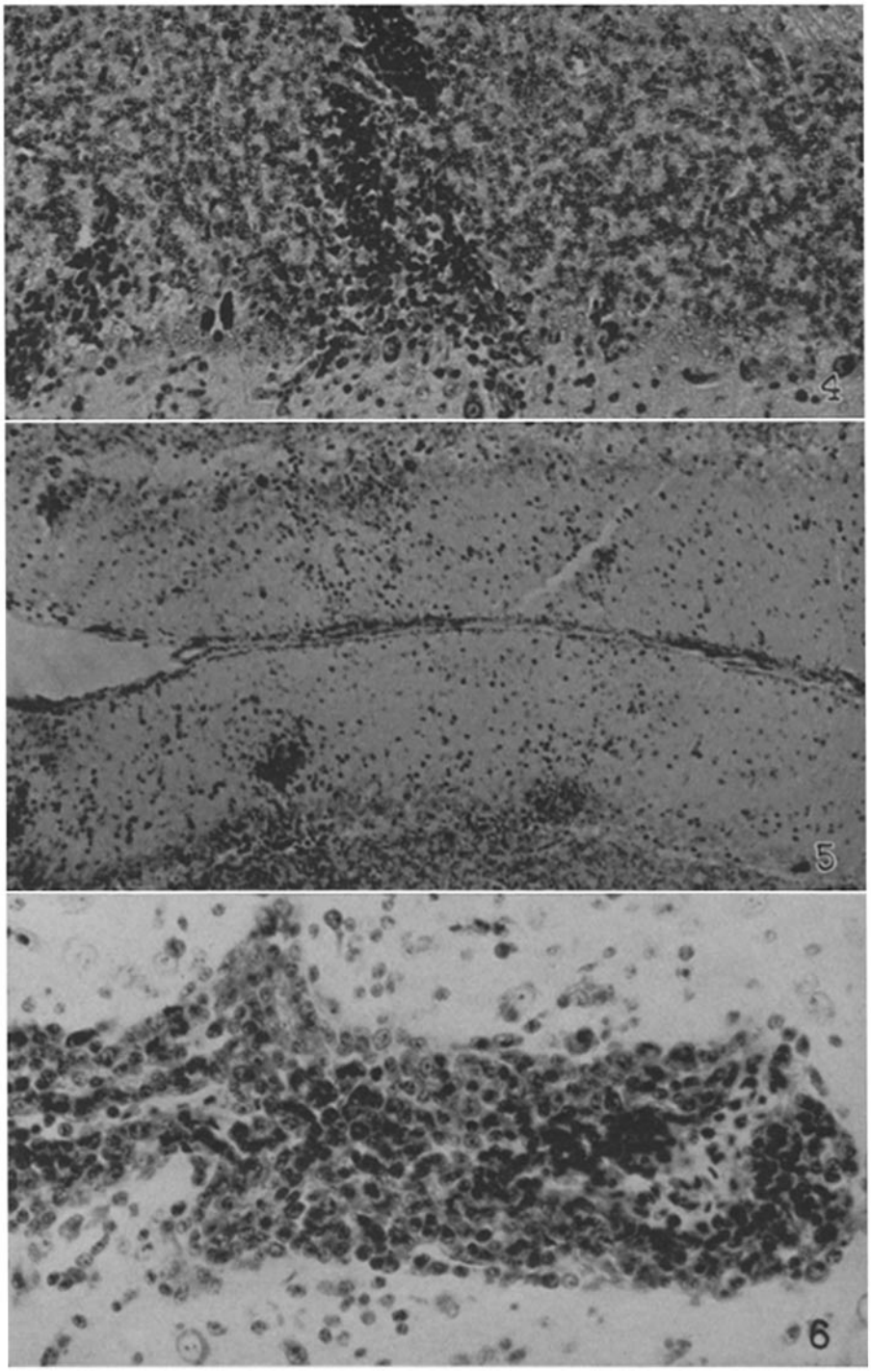
(Olitsky: Virus of infectious avian encephalomyelitis)

PLATE 44

FIG. 4. Perivascular lesion in cerebellum with good preservation of Purkinje's cells. Same chicken as in Fig. 3. × 250.

FIG. 5. Perivascular lesion in cerebellum and loss of Purkinje cells. Chicken 5-65 intracerebrally inoculated with avian virus. Incubation period 14 days; ataxic for 22 days. × 125.

FIG. 6. Detail of perivascular lesion in same chicken as Figs. 3 and 4. × 500.



Photographed by Joseph B. Haulenbeek

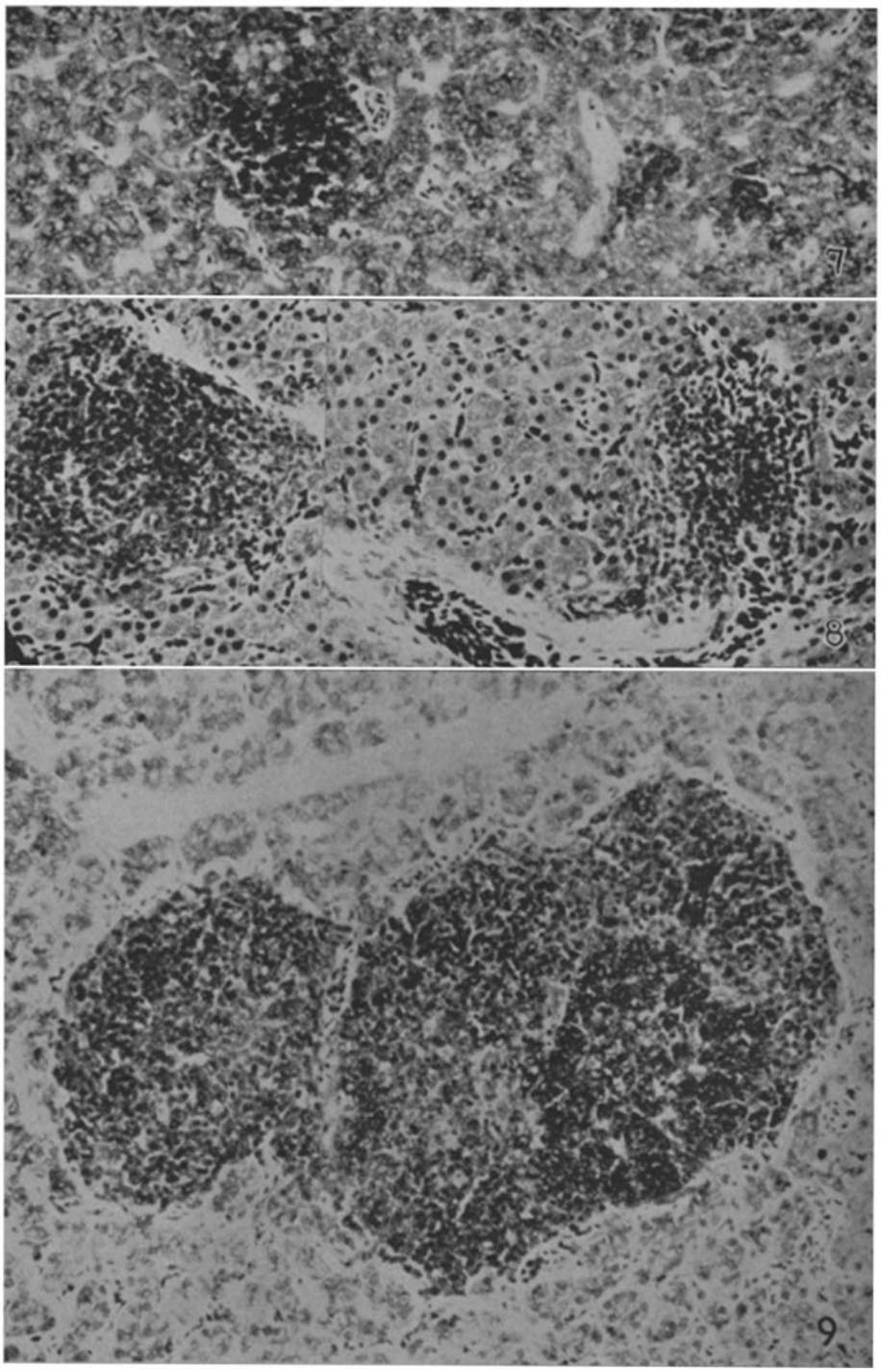
(Olitsky: Virus of infectious avian encephalomyelitis)

PLATE 45

FIG. 7. To be compared with Figs. 8 and 9. Liver of normal chicken showing normal lymphoid islands, one connected with blood vessel. These islands are somewhat larger here than are usually observed. × 275.

FIG. 8. To be compared with Fig. 7. Liver of chicken 3-22 intracerebrally inoculated with avian virus. Incubation period 12 days; clinical signs 1 day. To be noted is the hyperplasia of the lymphoid islands, the one at the right being associated with a blood vessel. × 275.

FIG. 9. Liver of chicken 6-09, subcutaneous inoculation of avian virus. Incubation 13 days; signs of experimental disease 1 day. Shows an extraordinary size of the area of lymphoid hyperplasia. × 275.



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(Olitsky: Virus of infectious avian encephalomyelitis)